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13. ABSTRACT (Maximum 200 Words)

Vascular endothelial growth factor(VEGF) is one of the most relevant mediators of capillary recruitment and stimulator of tumor-angiogenesis. Substantial evidence implicates VEGF in the vascularization of tumors, including those related to the mammary gland. Once synthesized and secreted by the normal mammary gland, VEGF is frequently bound to extracellular matrix molecules, remaining inaccessible to its receptors present on endothelial cells. Subsequent release of VEGF from extracellular sources is thought to require break-down of matrix proteins by matrix metalloproteinases(MMPs). However, its exact regulation is unknown. We previously found that MMP-3 cleaves $VEGF_{165}$(~22kDa) directly, releasing two major $VEGF_{165}$ cleavage products, ~16kDa and ~6kDa. Here we report that, by western analysis with epitope-specific antibodies, Edman sequencing and MALDI/MS analysis for both fragments to determine the cleavage sites in VEGF, MMP-3 cleaves $VEGF_{165}$, releasing the ~16kDa fragment that is functionally active as it phosphorylates VEGFR-2 in porcine aortic endothelial cells at a level comparable by conditioned media from uncleaved $VEGF_{165}$-expressing cells. The data imply that VEGF may be processed extracellulary releasing bioactive fragments and that this proteolysis might offer an important mode for regulation on VEGF bioavailability.

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Table of Contents

Introduction

VEGF-A is one of the most relevant mediators of capillary morphogenesis and stimulator of tumor-angiogenesis. VEGF-A exists in five forms, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{205}$, as a result of alternative splicing from a single gene. These various isoforms of VEGF-A differ in their affinity for heparin and extracellular matrix (ECM) proteins. The gene consists of 8 exons and all isoforms share exons 1-5 and exon 8. VEGF dimerization and receptor binding domains are encoded by exons 1-5 and the heparin and ECM binding domains are encoded by exons 6 and 7. Because of the heparin binding domain, all VEGF isoforms except for $VEGF_{121}$ rapidly bind to components of the ECM upon secretion, becoming non-accessible to its receptors located on endothelial cells: VEGFR-1, VEGFR-2 and neuropilin. Release of VEGF from the matrix is essential for stimulation of angiogenesis. It has been thought that VEGF may become available through cleavage of ECM proteins. However, the enzymes that mediate the release of matrix-sequestered VEGF remain to be determined. Recent findings from our laboratory revealed that VEGF might be cleaved directly by extracellular proteases releasing bioactive peptides, a finding that contradicts the dogma that VEGF is released "intact" through cleavage of extracellular matrix proteins. Although both might take place in vivo, our results would argue that VEGF is cleaved directly releasing bioactive fragments and that this proteolysis is an important mechanism involved in its regulation.

Key Research Accomplishments

In this Annual Summary Report I present the research accomplishments for the period of April 16, 2002 - April 15, 2003 under the Award number DAMD17-02-1-0328. This report addresses the research accomplishments with respect to the Statement of Work associated with specific Aims 1 and 2 : 1) to determine the MMP3 and MMP9 cleavage sites in VEGF and the sequence of the released VEGF peptides, 2) to determine the relevance of released peptides to VEGF receptor signal transduction.

Task 1. To determine the MMP3 and MMP9 cleavage sites in VEGF and the sequence of the released VEGF peptides:

1) **Determination of N-terminal sequence of VEGF peptide**
2) **Obtain mass spectrometry information of VEGF peptides**
3) **Characterization of VEGF peptides by western analysis**

Detailed studies on the proteolytic profile of VEGF revealed that this growth factor is not a substrate for MMP9, instead it is only cleaved by MMP3. We found small, however significant contamination of MMP3 in commercial preparations of MMP9 (MMP3 is used as activator of recombinant MMP9). This explains our preliminary findings. Therefore, we will pursue investigation on MMP3-mediated VEGF proteolysis as planned in the original proposal. We will also include plasmin-mediated proteolysis of VEGF for purposes of comparison. MMP3 cleaves $VEGF_{165}$ (~22kDa in SDS-PAGE under reducing condition) directly, releasing two major $VEGF_{165}$ species: a ~16kDa (for glycosylated VEGF, ~13kDa for unglycosylated) and ~6kDa fragments. In an attempt to determine the specific cleavage sites and the sequence of the released VEGF peptides, first, the VEGF cleavage products were subject to western blot analysis using epitope specific antibodies. The results indicated unequivocally that the species correspond to the N- and C-termini of $VEGF_{165}$, respectively (Fig. 1).

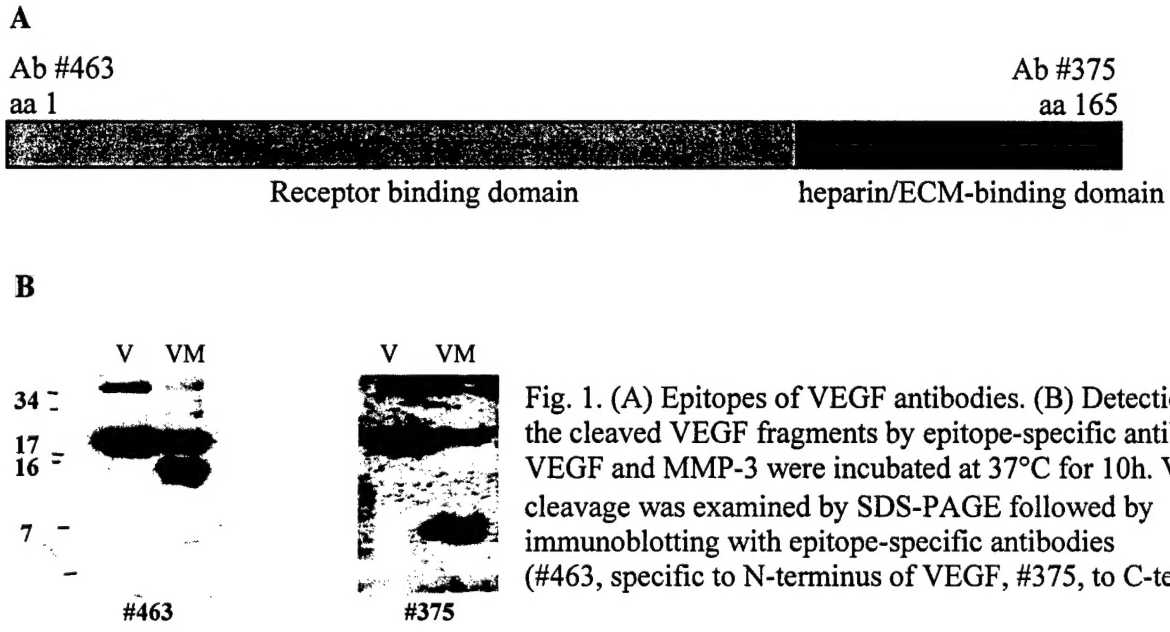

Fig. 1. (A) Epitopes of VEGF antibodies. (B) Detection of the cleaved VEGF fragments by epitope-specific antibodies. VEGF and MMP-3 were incubated at 37°C for 10h. VEGF cleavage was examined by SDS-PAGE followed by immunoblotting with epitope-specific antibodies (#463, specific to N-terminus of VEGF, #375, to C-terminus).

To identify the cleavage sites in VEGF, 1) the ~16 and ~6kDa fragments were separated by SDS-PAGE and were subject to N-terminal Edman sequencing, 2) the ~16kDa fragment was separated by SDS-PAGE, purified for MALDI/MS, LC/MS analysis, and digested followed by LC/MS/MS analysis. The results indicated existence of a proteolytically sensitive region proximal to the end of exon 5. Four cleavage sites were identified between aa 98-99, aa 113-114, aa 120-121 and aa 135-136.

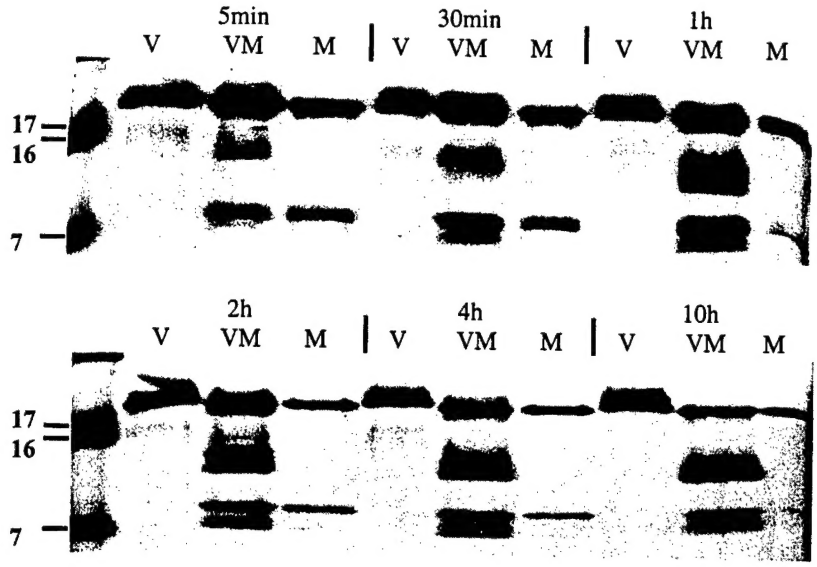

Fig. 2. VEGF cleavage by MMP-3 (time course). VEGF was incubated with MMP-3 at 37°C for different time points indicated as the above. VEGF cleavage was visualized by SDS-PAGE followed by silver staining. Unglycosylated mVEGF (Chemicon, Temecula, CA) was used.

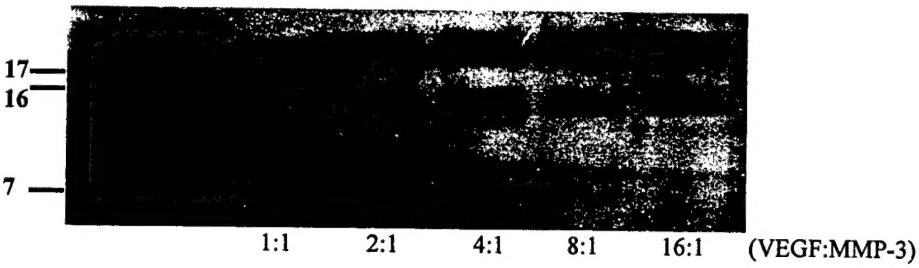

Fig. 3. VEGF cleavage by MMP-3 (dose response). Unglycosylated mVEGF was incubated with MMP-3 at 37°C for 4h with different molar ratios indicated as the above. VEGF cleavage was visualized by SDS-PAGE followed by silver staining.

Task 2. To determine the relevance of released peptides to VEGF receptor signal transduction:

1) Generation of expression vectors for VEGF peptide (~16kDa fragment)

2) Generation of stable cells overexpressing VEGF peptide

3) Determination of the ability of VEGF peptide to lead to receptor phosphorylation

4) Optimization of condition to identify ~16kDa VEGF peptides in vivo

An expression vector comprising VEGF 1-113 amino acids was generated and stably transfected into 293T cells to generate recombinant ~16kDa VEGF species. To test the ability of this truncated form in receptor phosphorylation, conditioned media from $VEGF_{1-113}$ expressing cells was incubated with porcine aortic endothelial cells overexpressing VEGFR2 (PAE-KDR). We found that the ~16kDa VEGF fragment is functionally active as it phosphorylates VEGFR-2 in porcine aortic endothelial cells at a level comparable to conditioned media from cells expressing full length $VEGF_{165}$ (Fig. 4). The effect of VEGF on endothelial cell proliferation, survival, angiogenesis and its binding to receptor is currently being tested by using purified peptide.

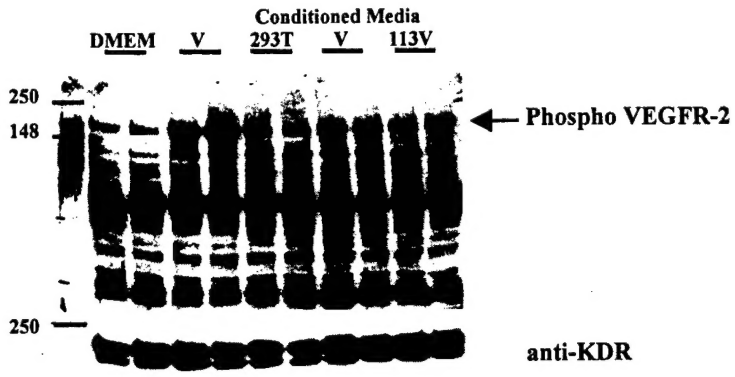

Fig. 4. Phosphorylation of VEGFR-2 (KDR) by VEGF fragment. PAE- KDR cells were incubated with purified VEGF, conditioned media from wild type VEGF and cleaved VEGF expressing cells. Phosphorylation of VEGFR-2 was examined by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine and anti-VEGFR-2 antibodies, respectively.

To identify MMP3-cleaved VEGF peptides in vivo, we first tested the battery of VEGF antibodies to immunoprecipitate the ~16kDa VEGF fragment and identified one polyclonal antibody (provided by Dr. Don Senger, Harvard University) that immunoprecipitated ~16kDa VEGF fragment from tumor lysates of T47D cells expressing full length mVEGF xenograft. Experiments to ascertain the presence of $VEGF_{113}$ in human tumors is underway.

Reportable Outcomes

Development of 293T and T47D (breast tumor) cell lines expressing $VEGF_{113}$

Conclusions

These results demonstrate that VEGF may be processed extracellularly releasing bioactive fragments and that this proteolysis might offer an important mode for regulation on VEGF bioavailability.

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